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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Molecular epidemiology can elucidate new breast cancer risk factors and gene-environment interactions relating to both hormonal and non-hormonal carcinogenic mechanisms. Corroborative epidemiological studies of intermediate biomarkers of carcinogenesis and laboratory studies demonstrating functional importance of the epidemiology findings are needed. The study of carcinogen-DNA adducts can provide corroborative evidence for the importance of genetic susceptibilities in breast cancer risk. We are establishing new assays for the detection of carcinogen-DNA adducts, use it for the first time in humans, and rigorously validate it to prove its utility for human breast tissue analysis in epidemiological studies, and determine adduct levels in relation to metabolizing gene polymorphisms. The originally proposed assay is novel because one uses a new chemical postlabeling method and quantitates adducts by accelerator mass spectroscopy (an ultrasensitive ^{14}C detection unit). We are now also using an enzymatic postlabeling method that will still rely upon ^{14}C AMS detection, but is specific for 4-aminobiphenyl. We are also continuing to develop a capillary HPLC and laser-induced flourescence for polycyclic aromatic hydrocarbons. Once validated, we will learn the variability for DNA adduct levels in the population as it relates to age, gender, race, and smoking in breast tissues from 235 donors (200 women, 35 men). In order to understand the determinants of DNA adduct formation in the breast tissue, we also have been identifying cytochrome P450 immunostaining in the same tissues. Concurrently, we have been collecting and using cultured breast strains from normal donors to determine in vitro adduct formation levels and correlate these levels with p53 induction. Thus, this study will provide new information about genotype-phenotype relationships.

14. SUBJECT TERMS
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(4) Introduction

Molecular epidemiology can elucidate new breast cancer risk factors and gene-environment interactions relating to both hormonal and non-hormonal carcinogenic mechanisms. Currently, many ongoing breast cancer studies are exploring risks related to genetic polymorphisms in these genes. Yet these studies by themselves do not provide absolute proof of etiology or causality. Thus, corroborative epidemiological studies of intermediate biomarkers of carcinogenesis and laboratory studies demonstrating functional importance of the epidemiology findings are needed. We are focusing on carcinogen-DNA adducts because they are promutagenic and lead to alterations in cancer susceptibility genes. They also serve as a marker of the biologically effective dose of a carcinogen, indicating a person's phenotype for metabolism, DNA repair and apoptosis. Several available carcinogen DNA-adduct assays might be useful here, but they are not sufficiently specific and/or sensitive for testing mechanistic hypotheses in humans. We are attempting to establish a new assay for the detection of carcinogen-DNA adducts, use it for the first time in humans, and rigorously validate it to prove its utility for human breast tissue analysis in epidemiological studies. This assay is novel because it uses a new chemical postlabeling method and quantitates adducts by accelerator mass spectroscopy (an ultrasensitive ^{14}C detection unit). We will develop and rigorously validate the assays using benzo(a)pyrene- (BPDE) and 4-aminobiphenyl (4-ABP)-related adducts as prototypes. Other methods are being explored in the event that finalization of the proposed method is not possible or if other, less labor intensive methods, can be done. With the development of an assay, we will learn the variability for DNA adduct levels in the population as it relates to age, gender, race, and smoking in breast tissues, and explore relative levels in liver tissue from autopsy donors. From a subset, 30 matched blood samples will be used to determine the relationship of breast levels (i.e., the target organ) to blood (i.e., the surrogate tissue). Finally, in these subjects, we will perform assays for genetic polymorphisms, to assess the association of "at risk" genetic variants with higher breast adduct levels. We also will test genotype-phenotype relationships by culturing primary breast cells from the same women, correlate adduct levels from in vitro carcinogen exposure and determine p53 response. This will allow us to establish the variability in the population for p53 induction from carcinogens, and might imply an independent risk for women with a low response.

During the interim between awarding the grant and receiving the funding, Dr. Shields decided to move his research laboratory from the intramural program of the National Cancer Institute to the Lombardi Cancer Center (LCC) of Georgetown University, effective January 1, 2000. Thus, it was decided to delay the implementation of the project until the budget and project design could be reorganized. First, it was inefficient to begin the project at NCI, which would last only three months and then require an interruption of the work and change in personnel. Upon arriving to the LCC, there was additional time needed to hire a postdoctoral fellow and establish the laboratory, although some work began almost immediately by Drs. Shields and Goldman. Thus, this project was not fulling implemented until about 6 months after the initial project date. All needed equipment, reagents and tissue samples were either transferred from the NCI, or purchased new at the LCC.

It should also be noted with the transfer of the grant from the NCI to LCC, there was a loss of available money because the NCI does not require overhead costs, while the LCC does. The DOD was requested to increase the cost of the total award to cover the additional overhead. This was denied and so there was a required rebudgeting to allow for the overhead, which reduced the available direct monies by almost 30%. Thus, some tasks may not be accomplished by the end of the three year grant period.

5) Body

Task 1: **To develop a ^{14}C-postlabeling method with acetic anhydride using micropreparative techniques for chemical specificity and AMS for sensitivity (CAP). BPDE and 4APB adducts will be used as prototypes. (Months 1 -18)**

As will be described, we had been simultaneously developing the CAP method for BPDE and 4ABP. And we began pursuing a capillary HPLC and laser induced fluorescence method (LIF) for the detection of BPDE adducts, because we expect that this will be simpler and less labor intensive. However, we have identified an alternate labeling methodology that will continue to rely on AMS, but use an enzymatic method for ^{14}C postlabeling. These two methods will be described below.

<u>^{14}C Postlabelling method for detection of 4-ABP adducts</u>:

We have been using the N-actyltransferase gene to postlabel 4ABP adducts with ^{14}C-labeled acetylCoa. This is then coupled with accelerator mass spectroscopy (AMS) for detection of the postlabelled adducts. Briefly the method the method involves digesting the sample DNA to yield a mixture adducted and non-adducted nucleotides. Purifying the adducted nucleotides by immunopurification procedure follows this. Subsequently the carcinogen-base adducts are subjected to an acylation procedure using ^{14}C-acetic anhydride. The products are isolated using HPLC followed by detection using AMS procedure. For this purpose, adducts of 4-aminobiphenyl with guanosine have already been synthesized by us. The authenticity of these materials has been confirmed during this grant period by mass spectroscopy.

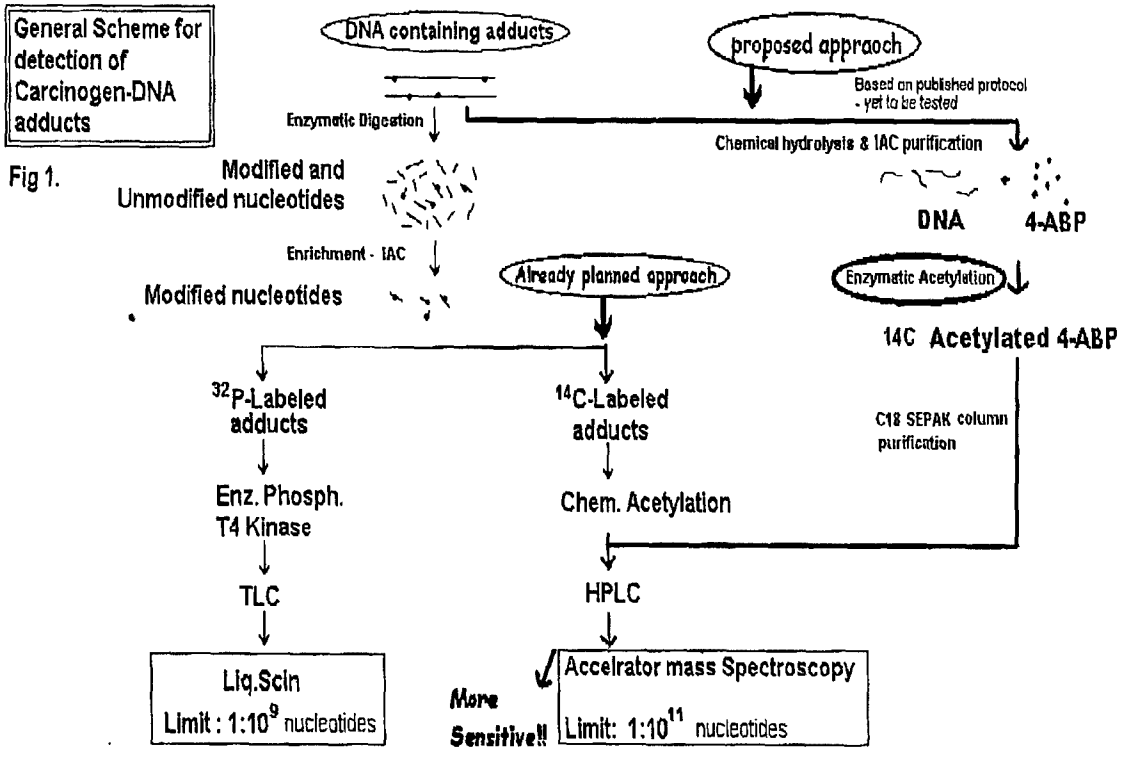


Fig 1.

We have adopted a two-fold approach to towards standardizing the detection of ABP-DNA adducts in patient samples. One is detecting ABP-guanosine adducts as such by obtaining digested products of DNA samples followed by immunopurification. Secondly, examine the possibility of hydrolyzing the 4-aminobiphenyl from the bases itself, thus trying to do away with process of immunopurification procedure (Figure 1). Also, this would make the methodology more specific. The present report is based on the second approach that has given promising leads. This has been explained more schematically in the schematic diagram presented below.

The proposed approach envisages the use of an enzyme N-acetyltransferase to incorporate the ^{14}C in the released 4-aminobiphenyl (after hydrolysis from DNA). This is distinctly different from the ^{32}P postlabelling approach, which also uses an enzyme. The specificity of the presently used enzyme to 4-aminobiphenyl (or any related arylamine) confers a unique advantage in detection of the adducts

This technique differs from

1. The enzymatic approach used in ^{32}P-postlabelling (**Fig 1** - faint highlight), in that the enzyme is targeted towards 4-aminobiphenyl alone rather than adduct. Hence the specificity for the enzyme on the substrate could remain relatively unaffected and be more amenable to enzymatic modification. The release of 4-aminobiphenyl from the DNA adduct would be achieved by simple alkaline hydrolysis based on an established protocol. Thus retaining the quantity of the carcinogen from the sample as unaffected as possible.
2. Either of the earlier approaches (i.e. ^{32}P-postlabelling or chemical ^{14}C-postlabeling) in that it does away with the necessity of enriching adducts in the sample (IAC – immunoaffinity chromatography). Though the IAC procedure can be used effectively in this approach too, the specificity of the enzymatic acetylation procedure would not warrant such a requirement.
3. Chemical postlabeling in that it hopes to do away with extensive cleanup procedure to wash out the excess labeled ^{14}C (also thereby preventing loss of sample

Standardizing the methodology involved the following important steps:

1. Test the hydrolysis of 4-aminobiphenyl from prepared 4-aminobiphenyl-DNA adducts (based on a protocol from an established hydrolysis procedure. This would demonstrate the successful release of 4-aminobiphenyl after the alkaline hydrolysis step.

    a) As a prelude to this procedure synthesize N-hydroxy-4-aminobiphenyl for preparing 4-ABP-DNA adducts. Once the hydrolysis step is established, the reaction would be advanced to use of tritiated N-hydroxy-4-aminobiphenyl. Synthesis would be by another established procedure.
    b) Prepare 4-ABP-DNA standards using calf thymus DNA
    c) Carry out alkaline hydrolysis of the adducted DNA and determine the efficiency of release from DNA.
    d) Standardize the hydrolysis procedure of 4-aminobiphenyl from already synthesized ABP-Gu adducts.

2. Normalize the enzymatic acetylation procedure of the starting material, 4-ABP.

a) Standardize the reaction conditions with respect to temperature, duration of reaction, and amount of acetyl CoA required for acetylation (reactions would be initially carried out using cold acetyl CoA).
b) Test the efficiency of recovery after subjecting to C18 SEPAK cleanup.
c) Check the elution times of the unreacted Acetyl CoA with respect to the acetylated 4-ABP to ensure non-bleeding of the unreacted compound into the product peak.
d) Use HPLC initially as means for detection of products. Once the profiles the compounds have been set, scale down the amount of starting material (4-ABP) and the amount acetic anhydride. Subsequently go in for standardizing using ^{14}C labeled acetyl CoA.

3. Validation of Binding of antibodies to 4-ABP: Though this step is not totally necessary due to the fact that the enzymatic method is specific to 4-aminobiphenyl, this step might still be a useful procedure to enrich 4-ABP alone from other possible arylamines in the DNA sample. Antibodies have been obtained from both Gerald Wogan. Bind the antibodies to columns (obtained commercially). Calibrate the binding capacity of both 4-ABP and ABP-Gu using CapLC with appropriate controls.

Following were the main results of the steps outlined above:

- *Validation of hydrolysis procedure:*

1. Preparation of 4-ABP-DNA standards was followed by an established procedure. Calf thymus DNA was used as the DNA source and N-hydroxy-4-aminobiphenyl was used to add it to DNA. Preparation of N-hydroxy-4-aminobiphenyl carried out by partial reduction of 4-Nitrobiphenyl. NMR spectra confirmed the synthesis of the compound. Since the compound was unstable it was kept stored under argon gas trap at –20°C - -80°C. Solutions of the compound were prepared in ethanol or methanol but were used as soon as they were prepared.
2. N-hydroxy-4-aminobiphenyl was added to DNA (already dissolved in a suitable solvent , so as to keep the ratio of the pre-carcinogen to DNA nucleotides at 1.8×10^{-2}. Though this was starting ratio, the final binding efficiency could be determined by release of 4-aminobiphenyl after successful hydrolysis. After an overnight incubation, unreacted –hydroxy-4-aminobiphenyl was extracted into an equal volume of hexane twice over. DNA was then precipitated by the phenol:chloroform procedure, redissolved in water and was then re-estimated to check for DNA recovery. A near complete recovery was achieved.

3. The adducted DNA was then subjected to an alkaline treatment (50 mM NaOH) in a glass tube and kept overnight at 130°C. The released 4-aminobiphenyl was then extracted into Hexane (two times) and then dried in a rotary evaporator. After redissolving in 1 ml 20/80 Acetonitrile/water mix, the solution was then analyzed by HPLC to detect for the presence of 4-aminobiphenyl peak.

4. As anticipated, there was a peak at 11 min - by an already established gradient profile of water/Acetonitrile (Fig 2 – see below) . This peak was well separated from that of the starting material (N-hydroxy-4-aminobiphenyl – Fig 3 – see below), which eluted at 8 min. The peak had spectra corresponding to 4-

aminobiphenyl (λ_{max} – 280 nm). Fractions containing this peak were pooled and dried and concentrated to analyze by Mass spectroscopy. A molecular weight of 169 corresponding to that of 4-aminobiphenyl was obtained thereby confirming the nature of the released compound.

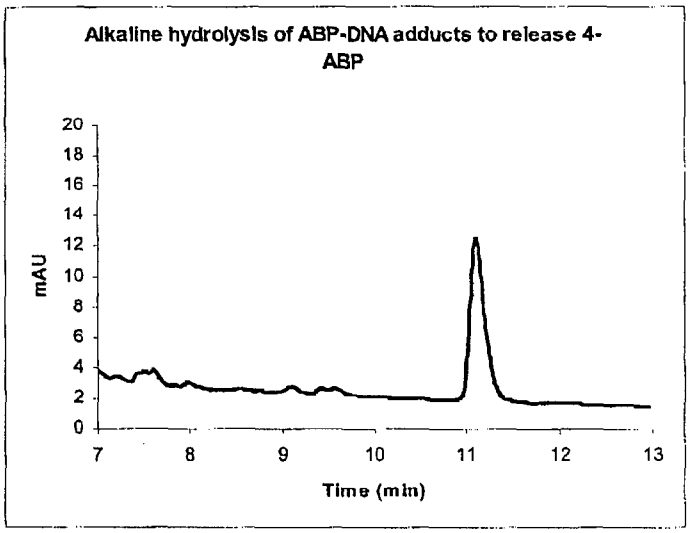


Fig 2

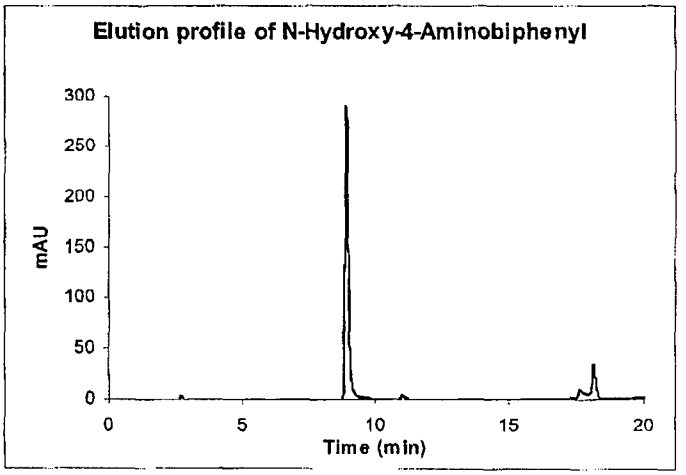


Fig 3

Thus, demonstration of the release of 4-aminobiphenyl made it possible to proceed further into examining the enzymatic acetylation process.

Normalization of the enzymatic acetylation procedure:

1. The enzymatic assay was performed using the enzyme N-acetyltransferase (E.C. 2.3.1.5) and Acetyl-CoA as the carbon donor. Unit definition of the enzyme was as defined by the supplier. The assay procedure was modified from the protocol provided by the commercial supplier (Sigma Inc). A typical reaction setup is described below

   Reagent A (100 mM Potassium phosphate buffer) – 60µl
   Reagent B (100 mM ß-mercaptoethanol) – 15µl
   Reagent C (30 mM EDTA) – 10µl
   Reagent D (4-aminobiphenyl) – 10µl (100µM – i.e. 1 nanomole of substrate)
   Reagent F (Enzyme solution in buffer – 100µl

   Mix well and split into two aliquots of 97.5 µl each

   Reagent E (Acetyl CoA) – 2.5µl (2.5µl water, if it is a blank)

   The reaction mix was incubated at room temperature for 20 min – 60 mins.

2. Following this the reaction mix was subjected to a cleanup using a SEPAK C18 column. The procedure was as follows

   1) Place the columns (one for a each reaction) over a vacuum manifold that are accordingly connected to the tube outlets inside the manifold.
   2) Wash once using methanol (~ 1-2 ml)
   3) Wash 3-4 times with water (~ 5 ml)
   4) Load the column with the reaction mix. Suck the contents into trash (already placed inside the manifold)
   5) Wash again with water 3-4 times
   6) Remove the trash container from within the manifold and replace with adjustable tube stand and place HPLC glass vials below each column.
   7) Load the column with a known amount of methanol and suck into tubes. Repeat the procedure 2-3 times with the total collected volume of methanol not exceeding 500-700µl.
   8) Dry the collected volume using speedvac rotor (if it is a reaction mix containing acetylated 4-aminobiphenyl or else dry using gentle air flow method if the collected volume is from a Blank sample (4-aminobiphenyl is unstable when subjected to drying by speedvac in glass vial.
   9) Redissolve in 100µl of HPLC run compatible solvent (20:80 ACN/H_2O mix) and analyze using HPLC (by the already established gradient profile).

3. In the reaction mix containing the acetylated product, there was an expected product peak at 9.5 min with a concomitant disappearance of the 11.0 min reactant peak (Fig 3). The reaction system containing the peak gave out unreacted 4-aminobiphenyl (11.0 min peak). Parallel controls using enzyme blank (no enzyme in reaction) were also carried out to rule out non-enzymatic conversion to

end product.

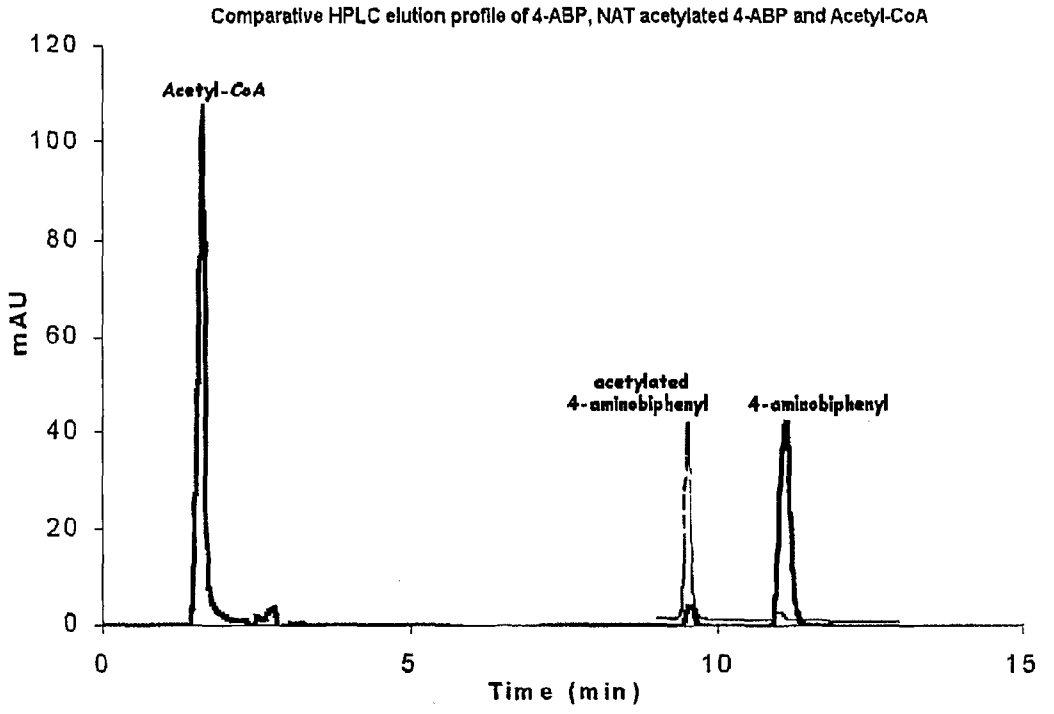


Fig 4

4. Subjecting the reaction mix to a C18 SEPAK cleanup resulted in a 70-75% recovery of the acetylated product.

5. Elution profile of acetyl-CoA alone showed the compound (Fig 4 - above) to be eluted out in the solvent front itself thereby resolving the separation of the reacted and unreacted peaks.

Future perspectives and plans:

1. Prepare ^{3}H ABP-DNA adducts standards (very low amount of ABP per DNA) for further validating hydrolysis procedure.
2. Ready to carry out ^{14}C labeling studies to test the adduct detection procedure using the newly outlined method.
3. Start on background cleanup work. This is the most important part of the assay as Accelerator Mass Spectroscopy is an ultrasensitive instrument to detect trace ^{14}C in samples.
4. Prepare labeled standards from tissue cultures and validate the assay.
5. Use human DNA sources for adduct detection
6. IAC step not necessary but if needed, hybridomas against 4-ABP have already been obtained.

Capillary liquid chromatography with laser induced fluorescence (capLC-LIF)

One of the best methods for B(a)P quantification is the fluorescent detection of B(a)P-tetraol following HPLC separation of the acid hydrolyzed B(a)P adduct. The B(a)P tetraol has strong fluorescence (λex=345 nm; λem=390 nm) which allows direct detection of the compound without labeling. We are taking advantage of this native fluorescence and further modifying the method to improve the detection sensitivity. To this purpose, we have recently installed a capillary LC system (Waters Inc., Milford, MA) together with a laser-induced fluorescence detector (Picometrics, Ramonville St Agne, France). The capLC decreases typical HPLC flow rates 1000 fold (from 1ml/min to 1µl/min). This promises an approximately 100 fold improvement in sensitivity over the HPLC/fluorescence when combined with the laser induced fluorescence detection (which can be focused on the capillary).

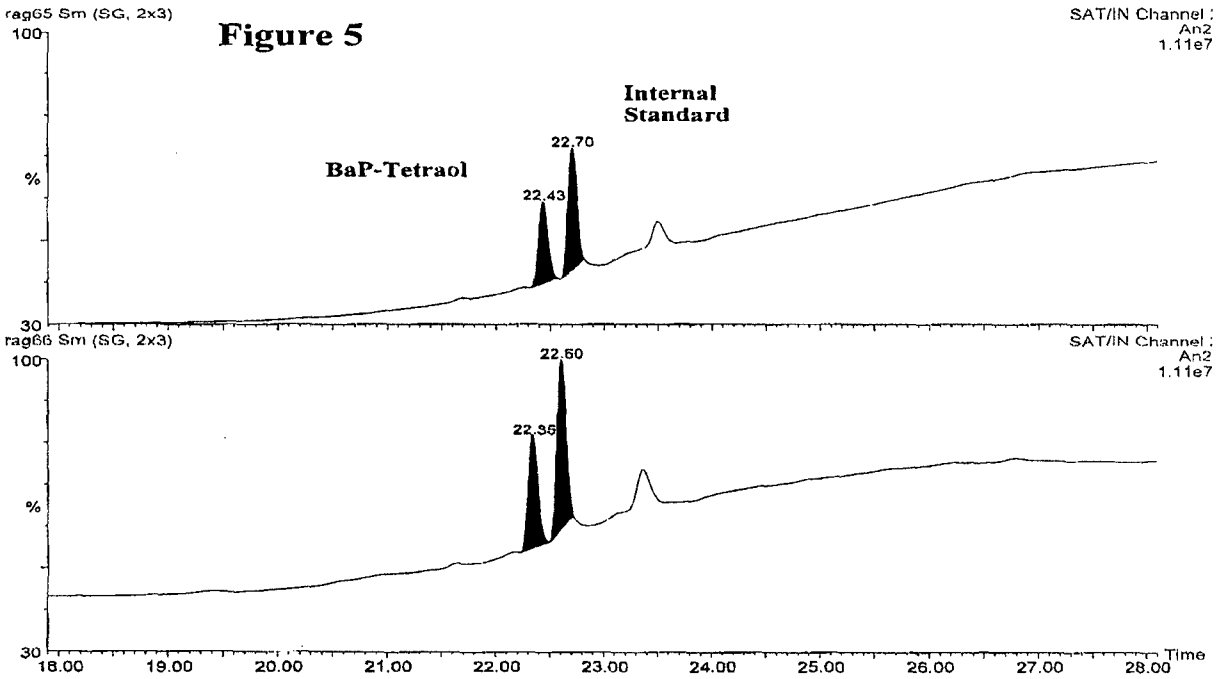
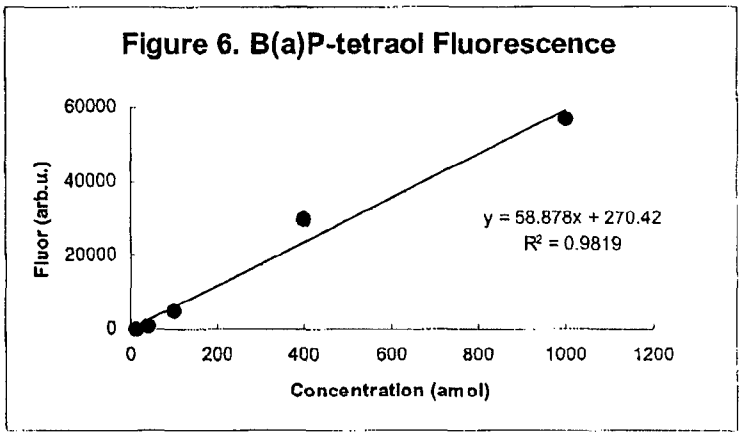


Figure 5

Both the capLC and the fluorescence detector are new instruments that were not available two years ago. And both instrument had technical problems that delayed somewhat our progress. The capLC had faulty valves causing inconsistent retention times. The Waters, Inc. replaced all three pumps on our capLC system. Since there are only few units produced each year and the demand for the instrument increases, the pumps were on back order for over six month. We have extensively validated the performance of the new pumps since the installation in June 2001. We have shown that the retention time is now reproducible within 0.1% which is consistent with the manufacturer specifications **(Figure 5)**.
This allows identification of the B(a)P by coelution at the retention time of a known standard.

The LIF detector was also damaged due to laser tube leakage. The laser head was repaired by Melles-Griot, Inc. (Carlsbad, CA). The repaired unit arrived in October 2001. We did verify that the sensitivity of the instrument was not affected. As the standard curve illustrates, we are

still able to detect the B(a)P tetraol with linearity over several orders of sensitivity down to 20 attomol of the standard **(Figure 6)**.

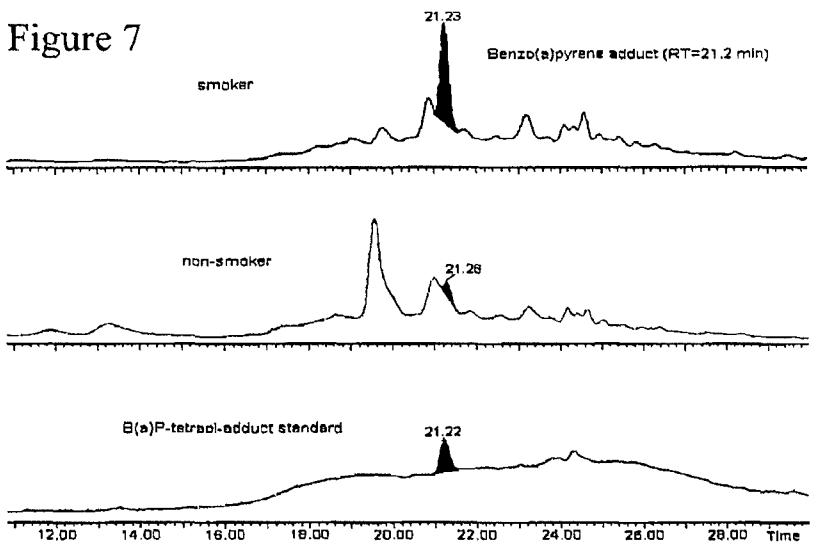


To improve the quantification of the B(a)P tetraol derived from human samples, we have incorporated an internal standard into the chromatographic procedures for adduct quantification **(Figure 7.)**. A number of standards were tested and the best standard was selected based on its fluorescence excitable at the 325 nm and its retention time under reverse phase chromatographic conditions. We have chosen a stereoisomer of B(a)P-tetraol (rtct) which does not form *in vivo* yet has properties similar to the B(a)P-tetraol (rttc). The retention time of the internal standard (22.7 minutes) is close to the retention time of the B(a)P-tetraol (22.4 minutes) yet is baseline separated from the analyte. The addition of the standard allows normalization of the adduct levels and improves quantification.

Two methods for sample cleanup prior to analysis were compared: 1. On-line traping of analyte on a precolumn with flow switching; and 2. Trapping of analytes on a seppak C18 cartridge. The on-line trapping requires use of the 3rd pump of the capillary LC system. This configuration increases the flow path and introduces unwanted variability in the retention time and broadening of the peaks. We have therefore focused on trapping of the B(a)P-tetraol on a C18 seppak cartridge (Alltech, State College, PA). The seppak is washed with 5ml of distilled water and the retained hydrophobic compounds including the B(a)P tetraol are eluted in 1ml of 100% acetonitrile and dried. The analytes are dissolved in 20% aqueous acetonitrole and separated by capillary LC using a 0.3x100 mm C18 column (Keystone Scientific, Bellefonte, PA).

We did verify the applicability of the method to human samples. We have analyzed two human samples with B(a)P adducts previously measured by 32P-postlabeling. One sample was lung tissue of a smoker with high B(a)P adduct level by 32P-postlabeling, the second lung sample was a nonsmoker with B(a)P adduct below the detection limit of the 32P-postlabeling method. 0.05 mg of each DNA was hydrolyzed by 0.1N HCl for 4 hours at 90°C. The DNA lysate was applied to a C18 seppak cartridge

Figure 7



(Alltech, State College, PA) and washed with 5ml of distilled water. The retained hydrophobic compounds including the B(a)P tetraol were eluted in 1ml of 100% acetonitrile and dried. The sample was reconstituted in 20μl of 25% aqueous methanol and injected into the capLC using an autosampler. The analytes were separated on a β-basic 100 x 0.3 mm column (Keystone Scientific, Bellefonte, PA) using a linear gradient of water and acetonitrile from 5% to 50% acetonitrile over 45 minutes. The B(a)P tetraol was identified by retenion time corresponding to the retention time of an authentic B(a)P standard (Chemsyn, Lenexa, KS). The result is shown in Figure 7. High adduct level by 32P-postlabeling was clearly higher by fluorescence (Figure). The sample undetectable by 32P-postlabeling was still measurable.

Task 2: To demonstrate the use of the new adduct detection method in human breast tissues, livers, and blood from autopsy and surgical reduction mammoplasty donors. (Months 18 - 36)

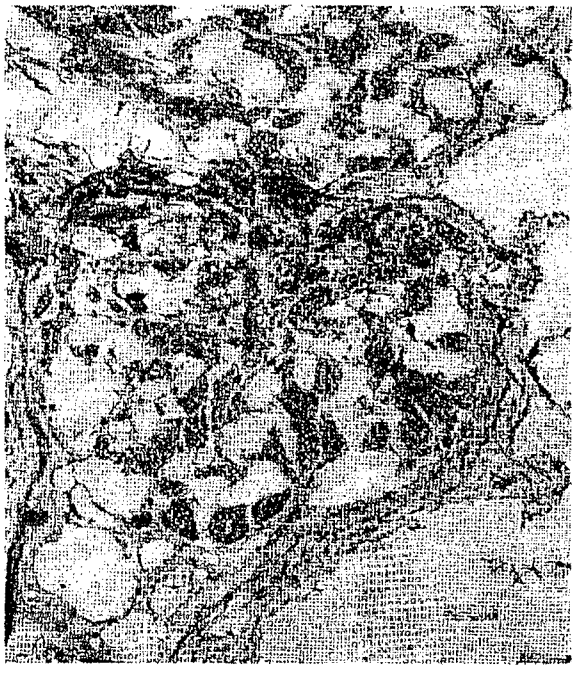
Autopsy samples as described in the grant proposal, collected at the NCI, are now at LCC. We have samples from matched breast, liver and lung samples. We also have samples from subjects who underwent reduction mammoplasty, and have developed primary strains from these same women. At the LCC, we have established the same study, where we recruit women who are undergoing reduction mammoplasty (about 1-2 per week), have them provide a 2 hour long personal interview, and provide us with breast tissue and blood. We then culture the tissue as well as bank fresh frozen tissue. Thus far, 28 subjects have been recruited.

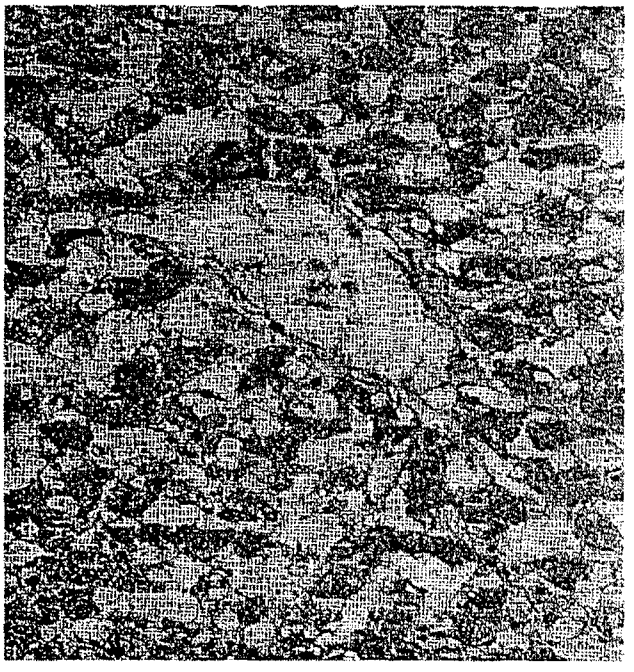
We have extracted DNA from the autopsy breast and reduction mammoplasty samples. The samples yielded at least 0.6 mg of DNA per gram of tissue. This quantity is in excess of our need to carry out the B(a)P adduct measurements (25ug per measurement). The excess DNA can be used for future expansion of the project to detect other adducts and related polymorphisms. We have purified the DNA by extraction with ethyl acetate and n-butanol (each in duplicate) to remove any unbound fluorescent impurities. These samples are ready for the hydrolysis and quantification of the B(a)P adduct.

We have shown that the proposed method for B(a)P-adduct detection is extremely sensitive (<100amol B(a)P-tetraol) and applicable to human samples. In the next year we will validate the method and quantify B(a)P adduct in the human samples.

Imunohistochemical Quantification of CYP1A1, CYP1B1, CYP3A

To better understand the carcinogenic pathway in smoking related lung and breast cancers, we are analyzing human autopsy tissues of smokers and non-smokers for parent polycyclic aromatic hydrocarbons, including benzo(a)pyrene, metabolic enzymes involved in activation of benzo(a)pyrene to the mutagenic benzo(a)pyrene diol epoxide, and the procarcinogenic B(a)P-DNA adducts. We have measured expression of the three major B(a)P activating enzymes, CYP1A1, CYP1B1, CYP3A in lung, breast, and liver tissues. This data will provide novel information and allow us to understand the mechanisms for DNA adduct formation.





CYP1B1 BREAST

CYP3A LIVER

So far we did analyze 17 breast and 25 liver samples and there are 15 matched pairs in lung/breast expressio the set. The summariz n is **1**. we are ed in **Table** analyzing also so that lung tissue we can compare and contrast results of an organ that clearly is susceptible to tobacco

TABLE 1

	Lung	Breast	Liver
CYP1A1	38/50	13/16	0/27
CYP1B1	45/50	14/16	3/27
CYP3A	44/50	8/16	26/27

carciongenesis. Most of the lung tissues express CYP1B1 and CYP3A, and 38 of 50 tissues express CYP1A1.

Future research will evaluate racial and gender differences in the CYP expression and induction by smoking, correlation of parent PAH compounds with expression of the metabolic enzymes, and correlation of DNA adducts of benzo(a)pyrene with the CYP enzyme expression.

Task 3: To use a novel breast primary epithelial culture system will be used to demonstrate the variability of p53 in the population. To determine p53 response in 40 human primary breast epithelial cells following exposure to benzo(a)pyrene (Months 30-36)

We have transferred 66 HMEC strains from the NCI. There is now an ongoing collection of tissues, with routine culturing at LCC (about 2 per month). p53 data in response to irradiation has been tested in 11 of those NCI strains and 9 of the LCC strains. The NCI strains were used initially to develop the protocol for p53 protein measurement, and once that was established, data was collected on strains that were subsequently unfrozen. Many of the experimental problems have been worked out, i.e. the best way to culture the frozen strains for p53 experiments, the amount of tissue I need from LCC patients to establish a strain. The main problem that still exists with the NCI frozen strains is that many did not survive the freezing process, however, since we have multiple tubes for each strain, we are hoping that at least one tube is viable, and we will be able to get data for each strain. If this fails, there is sufficient accrual at LCC, with fresh tissue, that the total number of subjects is not problem.

At LCC, tissue is collected from disease-free women undergoing reduction mammoplasty within 4 hrs. following surgery. The tissue is digested with a collagenase/hyaluronidase mixture into organoids. The organoids are plated in flasks following a 24 hour digestion period. The flasks are maintained in serum-free mammary epithelial cell growth medium and fed three times a week. Epithelial cells are passaged at confluency, which they typically reach 3-4 weeks following digestion. Frozen strains are thawed and maintained in the same manner. Experiments to measure p53 are done at the second or third passage. The cells are plated in equal amounts in 35 mm dishes at about 70% confluency (usually about 1 X 10^5 cells/per dish). Cells are then exposed to gamma-irradiation in a JL Sheperd Mark I irradiator at doses of 5, 10 or 20 Gy. Controls are sham-irradiated cells.

Four hours following irradiation, proteins are extracted from the cells and used in western analysis. p53 is measured using the DO(1) antibody according to standard protocols. Beta-actin protein levels are also measured. The results are quantitated using densitometry and the p53 protein levels are normalized to the beta-actin protein levels to obtain the level of p53 induction. In some experiments, 4-aminobiphenyl (4-ABP) was applied to cells cultures. A cytotoxicity assay was used to determine the appropriate concentration of 4-ABP and the cells were incubated in 0.3 uM, 30 uM (the ED_{10}), or 3mM 4-ABP. Non-treated cells were used for controls. P53 and beta-actin proteins were measured as above.

DNA damage resulting from chemical carcinogens and ionizing radiation has been shown to induce p53 in several experimental models. However, to our knowledge this has not yet been demonstrated in normal human mammary epithelial cells (HMECs). We have performed primary cultures of HMECs from six different cancer-free women undergoing reduction mammoplasties. The cells were passaged at confluency and experiments were performed at the third passage. The environmental carcinogen 4-aminobiphenyl (4-ABP) was prepared as a 10-fold dilution series with concentrations ranging from 30 mM to 30 nM and applied to the HMECs. Following a 24-hour incubation period, cell death was measured using a fluorescence assay. There was an increase of cell death at all concentrations of 4-ABP as compared to non-treated controls with a maximum two-fold increase in death with 30 mM 4-ABP. Using Western analysis, we have also shown an approximate two-fold increase in p53 protein in the HMECs of the one strain tested thus far with 0.3 uM 4-ABP. Additionally, we investigated the effect of γ-irradiation on the six HMEC cell strains. The HMECs were each exposed to γ-irradiation at

doses of 5, 10, and 20 Gy. p53 protein was detectable by Western analysis at all levels of irradiation, and at baseline in non-irradiated controls. An approximate 2-fold increase was demonstrated at the highest level of irradiation (20 Gy) from control levels (0 Gy) in all six strains. However, there was a differential amount of p53 induction among the strains at lower doses of γ-irradiation (5 and 10 Gy). The six strains can be separated into three distinct groups for response (none, low, and high) with three strains showing no significant increase in p53 at 5 and 10 Gy; two strains demonstrating a dose-dependent increase in p53 at 5 and 10 Gy; and one strain showing an initial 2-fold increase in p53 at 5 Gy and then leveling off at 10 and 20 Gy. These results indicate that the p53 response to DNA damaging agents differs in the breast and we hypothesize that such differences might influence breast cancer risk.

In order to ensure that the variability that we have observed was present in epithelial cells and that the differences were not due to differences in epithelial to fibroblast rations, we used cytokeratin staining by immunofluorescence to determine the percentage of fibroblasts contamination in human mammary epithelial cell cultures. The cultures expressed 80-99% epithelial cells after cytokeratin staining by immunofluorescence.

Separately, we assessed whether we can observe estrogen receptors in normal breast tissue. Using 6 frozen dissected tissues, we performed Western blots. We observed the ER alfa and beta expression in 5 of 6 samples for the tissue collected from reduction mammoplasty patients.

(6) Key Research Accomplishments:

1. The method for CAP detection of BPDE adducts is feasible, but there remains substantial problems in reducing background. One manuscript has been published.
2. We have identified a new method for radiolabeling 4ABP, using NAT2 and ^{14}C-acetyl coA. Initial results indicate that we will have both specificity and sensitivity.
3. Capillary HPLC and LIF is being developed for BPDE adducts. Preliminary data from human tissues indicates that this method is feasible. Calibration curves are now in progress to determine the limit of detection.
4. An epidemiological infrastructure has been established at LCC to continue accrual of women undergoing reduction mammoplasty.
5. Breast strains have been cultured, exposed to gamma radiation and 4-aminobiphenyl and differential p53 responses have been found. These cultures are greater than 90% epithelial. We hypothesize that women with better responses might be at reduced breast cancer risk.
6. Normal breast tissues from reduction mammoplasty donors indicates that these tissues have detectable ER alpha and ER beta.
7. Normal breast tissues from reduction mammoplasty donors express CYP1B1, CYP1A1 and Cyp3A.

(7) Reportable Outcomes

1. One manuscript has been published in the prior year.
2. Two abstracts have been submitted to the AACR. One describes the preliminary results for cultured breast strains and p53 response, and shows that these culture are epithelial. The other reports on the CYP immunostaining in breast, liver and lung tissues.
3. Tissue repository of breast tissues from women undergoing reduction mammoplasty has been established and collection is ongoing.

(8) Conclusions

This grant supports the development of BPDE and 4ABP adduct detection methods, which will be applied to breast tissues in order to examine genotype-phenotype relationships. It also supports the establishment and use of breast epithelial strains for examining the genotype-phenotype relationships and the p53 response based on adduct levels. Thus far, we have determined the basic CAP methods for the BPDE and 4ABP, but there are problems for the former with limitations in clean-up of contaminants. As an alternate strategy, we are developing a capillary HPLC and LIF method for BPDE adducts. The preliminary data in human samples is very promising, but the quantitative accuracy still needs to be determined. We also are using an alternative radiolabeling methodology for 4ABP adducts. We have established an epidemiological study and tissue repository of women undergoing reduction mammoplasty. In addition to the samples collected while at the NCI, this will ensure sufficient tissues for this study. We have identified CYP expression in the breast tissues, and also have confirmed ER alpha and ER beta expression. There is a clear variability in p53 response among women, and so we hypothesize that this might be related to breast cancer risk.

The initiation of this project was delayed at the start because Dr. Shields moved his laboratory, but the project has been fully implemented. Importantly, due to the change in institutions, less money remains available for research because of loss to indirect costs. Thus, it is not clear is all tasks will be completed, but thus far there is not yet reason to believe that this will be the case.